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Additional inventors are being named on the ______ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Nanoparticles and Their use for Multifunctional Bioimaging

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No. : UF-420P

Applicants : Swadeshmukul Santra, Paul H. Holloway, Robert A. Mericle, Heesun Yang

For : Nanoparticles and Their use for Multifunctional Bioimaging

MS PROVISIONAL PATENT APPLICATION
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DESCRIPTION

NANOPARTICLES AND THEIR USE FOR MULTIFUNCTIONAL BIOIMAGING

The subject matter in this application was made with government support under a research project supported by the Army Research Office Grant No. DAAD19-01-1-0603. The government may have certain rights in this invention.

Background of Invention

The human body is composed of tissues that are generally opaque. In the past, exploratory surgery was one common way to look inside the body. Today, physicians can use a vast array of imaging methods to obtain information about a patient. Some non-invasive imaging techniques include modalities such as X-ray, magnetic resonance imaging (MRI), computer-aided tomography (CAT), ultrasound, and so on. Each of these techniques has advantages that make it useful for observing certain medical conditions and parts of the body. The use of a specific test, or a combination of tests, depends upon the patient's symptoms and the disease being diagnosed.

MRI was established as a medical diagnostic technique that offers high-resolution anatomical information about the human body, and has since been used for the detection of a multitude of diseases. MRI creates images of a body using the principles of nuclear magnetic resonance. MRI can generate thin-section images of any part of the body from any angle and/or direction, in a relatively short period of time, and without surgical invasion. MRI can also create "maps" of biochemical compounds within any cross section of the body.

MRI is possible in the human body because the body is filled with small biological magnets--the most important, for MRI purposes, being the nucleus of the hydrogen atom, also know as a proton. Once a patient is placed into a MRI unit, their body is placed in a steady magnetic field that is more than 30,000 times stronger than the Earth's magnetic field. The MRI stimulates the body with radio waves to change the steady-state orientation of the protons,

causing them to align with the magnetic field in one direction or the other. The MRI then stops the radio waves and "listens" to the body's electromagnetic transmissions at a selected frequency. The transmitted signal is used to construct images of the internal body using principles similar to those developed for computerized axial tomography scanners (CAT scanners). Since the nuclear magnetic relaxation times of tissues and tumors differ, abnormalities can be visualized on the MRI-constructed image.

The continued use and development of MRI has stimulated interest in the development of contrast agents capable of altering MRI images in diagnostically useful ways. Contrast agents that are currently favored by researchers in the field are suitably complexed paramagnetic metal cations. The use of contrast agents in MRI imaging offers major opportunities for improving the value of the diagnostic information which can be obtained.

Radio contrast agents, which are used in radioisotopic imaging in a manner analogous to MRI contrast agents, are a well-developed field. The knowledge existing in this field thus provides a starting point for the development of MRI contrast agents. MRI contrast must meet certain characteristics, however, which are either not required or are considerably less critical in the case of radio contrast agents. MRI contrast agents must be used in greater quantities than radio contrast agents. As a result, they must not only produce detectable changes in proton relaxation rates but they must also be (a) substantially less toxic, thereby permitting the use of greater amounts; (b) more water soluble to permit the administration of a higher dosage in physiologically acceptable volumes of solution; and (c) more stable *in vivo* than their radiopharmaceutical counterparts. *In vivo* stability is important in preventing the release of free paramagnetic metals and free ligand in the body of the patient, and is likewise more critical due to the higher quantities used. For the same reasons, MRI contrast agents that exhibit whole body clearance within relatively short time periods are particularly desirable.

Since radio contrast agents are administered in very small dosages, there has been little need to minimize the toxicity of these agents while maximizing water solubility, *in vivo* stability and whole body clearance. It is not surprising therefore that few of the ligands developed for use as components in radio contrast preparations are suitable for use in preparation of MRI contrast agents. A notable exception is the well known ligand diethylene triamine

pentaacetic acid (DTPA), which has proved useful in forming complexes with both radiocations, pharmacologically suitable salts of which provide useful radio contrast agents, and paramagnetic cations such as gadolinium, whose pharmacologically suitable salts have proved useful as MRI contrast agents.

The contrast agents used in MRI derive their signal-enhancing effect from the inclusion of a material exhibiting paramagnetic, ferromagnetic, ferromagnetic, or superparamagnetic behavior. These materials affect the characteristic relaxation timers of the imaging nuclei in the body regions into which they distribute causing an increase or decrease in magnetic resonance signal intensity. There is therefore a long felt need for an MRI imaging agent which is substantially non-toxic, highly water soluble, and highly stable *in vivo* and which is capable of selectively enhancing signal intensity in particular tissue types.

Optical imaging continues to gain more acceptance as a diagnostic modality since it does not expose patients to ionizing radiation. Optical imaging is based on the detection of differences in the absorption, scattering and/or fluorescence of normal and tumor tissues. One type of optical imaging comprises near-infrared fluorescent ("NIRF") imaging. Generally, in NIRF imaging, filtered light or a laser with a defined bandwidth is used as a source of excitation light. The excitation light travels through the body and when it encounters a NIRF molecule or optical imaging agent, the excitation light is absorbed. The fluorescent molecule (*i.e.*, the optical imaging agent) then emits detectable light that is spectrally distinguishable from the excitation light (*i.e.*, they are lights of different wavelengths). Generally, light that is detectable via NIRF imaging has a wavelength of approximately 600-1200 nm. The optical imaging agent increases the target:background ratio by several orders of magnitude, thereby enabling better visibility and distinguishability of the target area. Optical imaging agents can be designed so that they only emit detectable light upon the presence of a particular event (*i.e.*, in the presence of a predetermined enzyme). Optical imaging, such as NIRF imaging, shows significant promise for detecting functional or metabolic changes, such as the overproduction of certain proteins or enzymes, in a body. This is useful because the majority of diseases induce early functional or metabolic changes in the body before anatomical changes occur. The ability to detect these

metabolic changes allows for early detection, diagnosis and treatment of a disease, thereby improving the patient's chance of recovery and/or of being cured.

A contrast agent is often used in conjunction with MRI and/or optical imaging to improve and/or enhance the images obtained of a person's body. A contrast agent is a substance that is introduced into the body to change the contrast between two tissues. Generally, MRI contrast agents comprise magnetic probes that are designed to enhance a given image by affecting the proton relaxation rate of the water molecules in proximity to the MRI contrast agent. This selective change of the T_1 (Spin-Lattice Relaxation Time) and T_2 (Spin-Spin Relaxation Time) of the tissues in the vicinity of the MRI contrast agents changes the contrast of the tissues visible via MRI. Generally, optical contrast agents comprise dyes designed to emit light when excited with outside radiation. This emitted light is then detected by an optical imaging device.

Contrast agents are administered to a person, typically via intravenous injection into their circulatory system, so that abnormalities in the person's vasculature, extracellular space and/or intracellular space can be visualized. Some contrast agents may stay in the person's vasculature and highlight the vasculature. Other contrast agents may penetrate the vessel walls and highlight abnormalities in the extracellular space or intracellular space through different mechanisms, like, for example, binding to receptors. After a contrast agent is injected into a tissue, the concentration of the contrast agent first increases, and then starts to decrease as the contrast agent is eliminated from the tissue. In general, a contrast enhancement is obtained in this manner because one tissue has a higher affinity or vascularity than another tissue. For example, most tumors have a greater MRI contrast agent uptake than the surrounding tissues, due to the increased vascularity and/or vessel wall permeability of the tumor, causing a shorter T_1 and a larger signal change via MRI.

Typical MRI contrast agents belong to one of two classes: (1) complexes of a paramagnetic metal ion, such as gadolinium (Gd); or (2) coated iron nanoparticles. As free metal ions are toxic to the body, they are typically complexed with other molecules or ions to prevent them from complexing with molecules in the body, thereby lessening their toxicity. Some typical MRI contrast agents include, but are not limited to: Gd-EDTA, Gd-DTPA, Gd-DOTA,

Gd-BOPTA, Gd-DOPTA, Gd-DTPA-BMA (gadodiamide), feruimoxsil, ferumoxide and ferumoxtran.

Another class of MRI contrast agents--called "smart" contrast agents--includes contrast agents that are activated by the physiology of the body or a property of a tumor, *i.e.*, agents that are activated by pH, temperature and/or the presence of certain enzymes or ions. Some examples of MRI smart contrast agents include, but are not limited to, contrast agents that are sensitive to the calcium concentration in a body, or those that are sensitive to pH.

"Smart" optical contrast agents have recently been used *in vivo* to monitor enzyme activity in the human body. These smart contrast agents only produce contrast in the presence of specific proteases. Since proteases are key factors involved in multiple disease processes, the ability to tailor contrast agents or probes to specific enzymes should ultimately allow one to detect the expression levels of marker enzymes for various pathologic conditions. This approach is capable of providing all the necessary information for studying pathologies near the surface of the skin via optical imaging. However, since low localization information is characteristic of optical imaging, one or more additional modalities may be required for diagnosing pathologies deeper within the body.

Contrast agents are not only useful, but are often times required in order to make the presence of certain diseases detectable. For example, the mechanisms of contrast in MRI (such as T_1, T_2 and/or proton density) are somewhat limited, allowing certain diseases to remain undetectable by MRI in the absence of exogenous contrast agents. This is because none of the parameters that influence contrast are affected in some diseases without the addition of a contrast agent. Therefore, using contrast agents in conjunction with MRI offers excellent sensitivity for detecting some additional pathologic conditions, thereby allowing some diseases to be detected that would otherwise be undetectable via MRI alone. For example, MRI in the presence of contrast agents has very high sensitivity for detecting breast tumors, but very low specificity for the detection of cancerous tissue. The specificity for identifying cancerous tissue is so low via MRI because multiple pathologies, such as the recruitment and production of new blood vessels (angiogenesis), are characterized by markers similar to those of cancerous tissue.

While both MRI and optical imaging provide useful information, neither independently provides all the information desired to help make early diagnoses of all diseases. As previously discussed, the majority of diseases induce early functional or metabolic changes in the body before anatomical changes occur. While these metabolic changes are almost impossible to detect via current MRI techniques, optical imaging shows significant promise in being able to detect such changes. However, when applications such as breast imaging are envisioned, optical imaging by itself is very limited by the spatial resolution that can be achieved.

Therefore, there is a need for systems and methods that can be used to further aid in the early detection of disease. There is also a need for systems and methods that allow for high-resolution localization of biochemical activity in a living organism. There is also a need for bifunctional or multifunctional contrast agents that can be utilized in two or more different modalities concurrently or consecutively. There is yet a further need for multifunctional contrast agents that can be utilized in both MRI and optical imaging concurrently.

Brief Summary of the Invention

The present invention relates to multifunctional contrast agents or probes, and methods of using the same. The invention provides fluorescent, radio-opaque and magnetic quantum nanoparticles (NP) for multifaceted bioimaging (*e.g.*, intra-arterial pre-operative brain mapping and broad based *in vivo* diagnostic imaging).

The present invention provides contrast agents and methods that allow high-resolution *in vivo* imaging of the localization of biochemical activity in a living organism, and are particularly useful in the early detection of disease. Embodiments of the present invention may comprise multifunctional contrast agents that can be utilized in two or more different imaging modalities concurrently or consecutively. In a preferred embodiment, the multifunctional contrast agent is a yellow-emitting monodisperse, ultra-small (*e.g.*, less than 10 nm), water-soluble CdS:Mn/ZnS nanoparticle (zinc sulfide capped and manganese doped cadmium sulfide quantum dot). Furthermore, the contrast agent is preferably coated with a systemically non-toxic material, such as amorphous silica. The nanoparticles of the present invention (also referred to herein as NP, quantum dots, Qdots, QD, probes, nanocrystals, detection agents, and/or contrast agents) are

superior to conventional fluorescent dyes because of their remarkable photostability, brightness, and suitability for *in vivo* multifaceted bioimaging.

In one embodiment, the multifunctional contrast agent of the present invention may be utilized concurrently in MRI and at least one other bioimaging modality, such as fluorescence imaging or CT scan (*e.g.*, angiography), simultaneously or consecutively in any order.

In another embodiment, the present invention provides multifunctional contrast agents that are designed to allow simultaneous or consecutive imaging of a target area using multiple modalities (*e.g.*, MRI and CT scan) so as to allow both anatomical and functional (*e.g.*,, metabolic) information to be obtained contemporaneously. The anatomical information can be obtained via MRI imaging, and the functional/metabolic information is obtained via another modality, in conjunction with the administration of a multifunctional contrast agent of the invention.

The multifunctional contrast agents of the present invention allow enhanced anatomical information to be obtained. As used herein, the term "enhanced" means that the image or information obtained by using the multifunctional contrast agent is of improved quality over the image or information that would be obtained by using no contrast agent. The enhanced anatomical information may be obtained via computed tomography, positron emission tomography, or magnetic resonance imaging, for example. The enhanced functional information may be obtained via near-infrared fluorescence imaging, for example.

In another aspect, the invention provides a method of obtaining *in vivo* imaging of biochemical activity in a body, comprising the steps of administering a multifunctional contrast agent of the present invention to a subject; obtaining an image of anatomical information of the subject; and/or obtaining an image of functional/metabolic information of the subject. Preferably, the subject is a living subject. These images may be obtained concurrently or consecutively in any order. In one embodiment, the multifunctional contrast agent is administered intravenously, but it may also be administered in any other suitable manner such as orally or intramuscularly. In some embodiments, the image of anatomical information may be obtained via computed tomography, positron emission tomography, or magnetic resonance imaging. In other embodiments, the image of functional information may be obtained via optical

imaging. Optionally, the method can further comprise recording the image in a tangible or computer readable (*e.g.*, electronic) medium, and/or diagnosing the subject based on the image obtained.

Brief Description of Drawings

Figure 1 shows XPS survey scans of (a) CdS:Mn, (b) ZnS:Mn, and (c) CdS:Mn/ZnS core/shell nanocrystals.

Figures 2A and 2B show transmission electron microscope (TEM) images of CdS:Mn/ZnS nanocrystals with a mean particle size of 3.1 nm and x-ray diffraction (XRD) patterns, respectively. The length bar at lower left corner in Figure 2A indicates 5 nm. The inset in Figure 2A is a nanocrystal viewed along the [001] zone axis and $(1\bar{1}0)$ and (110) planes are intersecting at 90 °.

Figures 3A and 3B show PL emission spectra of *n*-dodecanethiol- and ZnS-passivated CdS:Mn nanocrystals, respectively.

Figure 4 shows a comparison of PL brightness from *n*-dodecanethiol- and ZnS-passivated CdS:Mn nanocrystals under 366 nm UV irradiation.

Figure 5 shows variations of PL emission intensity of *n*-dodecanethiol- and ZnS-passivated CdS:Mn nanocrystals versus time exposed to 400 nm UV light. The monitored wavelengths are 580 nm and 585 nm for *n*-dodecanethiol- and ZnS-passivated CdS:Mn nanocrystals, respectively.

Figure 6 shows XPS spectra of core/shell nanocrystals after 366 nm UV irradiated for 3 hours in either air and Ar. The large peak at 173.8 eV for irradiation in air suggests formation of $ZnSO_4$.

Figure 7 shows variation of PL emission intensity of *n*-dodecanethiol-capped CdS:Mn/ZnS nanocrystals versus time exposed to 350 nm UV light. The monitored wavelength is 585 nm.

Figures 8A–8C show the magnetic field versus magnetization of Mn-doped core/shell quantum dots, measured at 10 K (Figure 8A), 100 K (Figure 8B), and 300 K (Figure 8C).

Figures 9A and 9B show the magnetic field versus magnetization of undoped core/shell quantum dots, measured at10 K (Figure 9A) and 300 K (Figure 9B).

Figures 10A and 10B show branches of the right middle cerebral artery of rats following endovascular injection of 10 mg/ml TAT-grafted quantum dots (CdS:Mn/ZnS) of the invention. The duration of the injection was three minutes. Figure 10A shows a micrograph of a cross section (rostral) of rat brain (10X magnification). Figure 10B shows a fluorescence micrograph of the same cross section shown in Figure 10A.

Figure 11 shows a higher magnification of the fluorescence image shown in Figure 10B.

Figure 12 shows cross sections of blood vessels labeled with the quantum dots of Figures 10A and 10B.

Detailed Disclosure of the Invention

The present invention provides multifunctional detection agents that, by virtue of their fluorescent, radio-opaque, and paramagnetic properties, function as contrast agents using one or more imaging modalities. These multifunctional detection agents aid in the detection of physiological changes associated with biochemical changes in the tissue, which may indicate tissue abnormality, cardiovascular disease, thrombosis, cancer, *etc.* Use of the multifunctional detection agents of the invention allow precise, direct, real-time visualization of normal and abnormal anatomical features. In a preferred embodiment, the multifunctional detection agent (contrast agent) is a yellow-emitting monodisperse, ultra-small (less than 10 nm), water-soluble CdS:Mn/ZnS nanoparticle (zinc sulfide capped and manganese doped cadmium sulfide quantum dot; also referred to herein as QD). The photoluminescent and electroluminescent properties of QD have been described by Yang H. and P. Holloway (Yang H. and P. Holloway, *Journal of Applied Physics*, 2003, 93(1):586-592; Yang H. and P. Holloway, *Applied Physics Letters*, 2003, 82(12):1965-1967), are incorporated herein by reference in their entirety. The contrast agents of the invention can also be used to image non-biological opaque mediums.

The contrast agents of the present invention exhibit fluorescent, radio-opaque, and paramagnetic properties, allowing imaging by photoluminescence, fluoroscopy, and MRI, all using the same semiconductor crystalline material. The contrast agents of the present invention

are superior to conventional fluorescent dyes because of their remarkable photostability, brightness, and suitability for *in vivo* multifaceted bioimaging (*e.g.*, fluorescence, CT scan/angiography, MR imaging/angiography). Thus, advantageously, the multifunctional contrast agents of the present invention can function as both MRI contrast agents and optical contrast agents. As used herein, the term "MRI contrast agent" or "magnetic resonance contrast agent" means a molecule that can be used to enhance an MRI image. As used herein, the terms "optically detectable agent" and "optical contrast agent" mean a photoluminescent compound (*i.e.*, a compound that will emit detectable energy after excitation with light).

The contrast agent of the invention is preferably water soluble. The particles can be made or rendered water soluble using various techniques, such as surface modification. For example, the contrast agent can be coated with a systemically non-toxic material, such as amorphous silica. However, other strategies to water-solubilize quantum dots with a hydrophobic surface can be utilized. For example, mercaptoacetic acid can be used as a coupling reagent with ZnS capped CdSe (CdSe/ZnS) quantum dots (Chan, W. C. W., and Nie, S. M. *Science* 1998, 281:2016). Other methods that may be utilized include encapsulation of individual quantum dots with an amphiphilic polymer (Wu, X. Y. *et al.*, *Nat. Biotechnol.* 2003, 21:41; Larson, D. R.; Zipfel, W. R.; Williams, R. M.; Clark, S. W. *et al.*, *Science* 2003, 300:1434), micelle-forming hydrophilic polymer-grafted lipids (Dubertret, B. *et al.*, *Science* 2002, 298:1759), or biodegradable polymers such as polylactic acid (PLA), polyglycolic acid (PGA), PLGA (PLA-co-PGA), dextran, and alginate.

In another aspect, the present invention provides compositions comprising the multifunctional agents of the present invention in admixture with at least one pharmaceutically acceptable carrier, as diagnostic agents and/or therapeutic agents. For instance, these active ingredients are useful for the manufacture of medicaments suitable for imaging or imaging-aided applications, including MRI, nuclear scintigraphy (NS), MRI-aided applications or NS-aided applications or for the manufacture of imaging agents or imaging-aided agents for use in such applications. This includes their use as *in vivo* effective contrast agents, including multipurpose contrast agents, for visualizing and/or identifying organs, parts of organs or systems such as for example the vasculatory system, the hepatobiliary system or the renal-urinary system, tissues

such as for example necrotic tissue, and for visualizing and/or identifying diseases and pathologies.

Diseases that may be visualized and/or identified using the multifunctional contrast agents of the invention include, but are not limited to, ischemic insults such as myocardial or cerebral infarction and space-occupying lesions (*e.g.*, tumors or inflammatory lesions) in solid organs such as the liver, kidney, spleen, adrenal gland, *etc.* These agents are also useful in the follow-up of a therapy, for instance the evolution of necrosis. In particular, these contrast agents are useful in medical applications involving necrosis and necrosis-related pathologies, such as pathological or therapeutic necrosis caused by pathologic or therapeutically-induced ischemia or originating from trauma, radiation and/or chemicals, including therapeutic ablation, radiotherapy and/or chemotheraphy, myocardial and cerebral infarctions. For this purpose, they are administered to the human body, preferably enterally or parenterally, as therapeutic and/or diagnostic agents.

Using the contrast agents of the present invention, endovascular pre-operative brain mapping techniques will produce a visual contrast between eloquent (functional) and non-eloquent ("silent") brain tissue. The significance of this approach is to individualize the unique brain functional map of each patient requiring brain resection to improve their neurosurgical outcome. This technique will be useful for malignant glioma resection, low-grade glioma resection, arteriovenous malformation resection, brain resection for epilepsy surgery, and other functional neurosurgical resections. It will improve upon current techniques by allowing a precise, direct, real-time visualization of non-eloquent/silent brain tissue that can be resected with absolute assurance that no neurological deficit will result. Targeting moieties may also be used selectively to direct the accumulation of the contrast agents of the present invention. For example, upon administration via microcatheter under gluoroscopic guidance, HIV TAT-grafted contrast agents of the present invention will be either internalized rapidly by the brain capillary endothelial cells (via endocytosis/pinocytosis), or will be strongly adsorbed onto cell membranes resulting in staining of that portion of the brain. The post-staining follow-up with CT-scan and/or MRI scan will confirm the NP distribution (mapping) prior to surgery.

For diagnosis purposes, nanoparticles of the invention can be grafted with folic acid as a targeting moiety, to specifically target the folate-receptor over-expressing metastatic adenocarcinoma. Alternative delivery methods include catheter-assisted administration of HIV TAT peptide grafted NP probes through tumor feeding arteries. This technique will not only allow surgeons to acquire CT/MRI scans non-invasively but also will guide them for effective resection of lesions in real time through NP fluorescence.

In conjunction with the contrast agents of the present invention, an HIV TAT-mediated endovascular brain mapping technique will be useful for malignant glioma resection, low-grade glioma resection, arteriovenous malformation resection, brain resection for epilepsy surgery, and other functional neurosurgical resections. This method will improve upon current techniques by allowing a precise, direct, real-time visualization of non-eloquent/silent brain tissue that can be resected with absolute assurance that no neurological deficit will result from the neurosurgical resection.

Many metastatic adenocarcinoma *e.g.*, breast adenocarcinoma, lung adenocarcinoma, oral carcinoma and pituitary adenoma overexpress folate receptors. There are many clinically challenging brain tumors such as malignant gliomas that may also possibly over-express folate receptors and can be effectively targeted using the contrast agents of the present invention. For example, if substantial amounts of nanoparticles are present due to the folate receptors, the tumor will glow (emit fluorescent light) when exposed to an excitation photon source. Malignant tissue can be clearly distinguished from normal tissue in real time to delineate malignancies from effective surgery.

The multifunctional contrast agents of the present invention can be synthesized using a water-in-oil (W/O) microemulsion method. The nano-size water droplets serve as the nano-reactor. Several factors, including water-to-surfactant molar ratio, type of surfactant, and co-surfactant molecules and their ratio, reaction type and nature of active agents, can be varied to affect the size and surface functionality of the nanoparticles of the invention.

Pharmaceutically acceptable carriers for use in admixture with the contrast agents of this invention are well known in the art of pharmacy and will be selected depending on the mode of administration to the patient (*i.e.*, the mammal, in particular humans) involved. Typically, a

suitable formulation is a physiologically acceptable liquid formulation, preferably an aqueous solution or an emulsion or suspension including conventional surfactants such as polyethylene glycol.

In another aspect, the invention relates to a method for generating an image of at least a part of the body of a subject, such as a human or non-human animal, comprising systemically or locally administering to the subject an effective amount of a contrast agent of the present invention. Preferably, the contrast agents of the invention are used systemically as diagnostic agents by parenteral administration, such as intravenous injection, at low doses. Optionally, the method can further comprise recording the image in a tangible or computer readable (*e.g.*, electronic) medium, and/or diagnosing the subject based on the image obtained.

The contrast agents of the invention are also useful for local administration, *e.g.*, including intracoronary administration in the case of a patient with myocardial infarction.

The present invention also comprises methods of obtaining high-resolution, *in vivo* images of biochemical activity in a subject. Preferably, the subject is a living subject. One method comprises estimating the localization of the contrast agent using one modality (*e.g.*, MRI), while concurrently estimating the level of biological activity using a second modality (*e.g.*, optical imaging). Another method comprises obtaining an image of anatomical information of a living organism and obtaining an image of functional information of the living organism, wherein a multifunctional contrast agent of the invention is introduced within the living organism. The multifunctional contrast agents of the present invention may be administered in any suitable way, preferably via intravenous injection. Optionally, these methods can further comprise recording the image in a tangible or computer readable (*e.g.*, electronic) medium, and/or diagnosing the subject based on the image obtained.

The multifunctional detection agents of the present invention allow both anatomical and functional information to be obtained simultaneously via at least two different modalities. Using a first modality (*e.g.*, magnetic resonance imaging) provides high-resolution anatomical information from which the precise anatomical localization of the detection agent can be determined. Using a second modality (*e.g.*, optical imaging) provides functional or metabolic information. This combination of anatomical and functional information allows for easier and

earlier diagnosis and treatment of diseases than currently exits, thereby improving the patient's chance of recovery and/or of being cured.

While multifunctional detection agents for concurrent or consecutive use in combination with MRI/optical imaging systems have been described above, it is understood that multifunctional detection agents may be designed for concurrent or consecutive use in alternative combination imaging systems without deviating from the scope of the present invention. For example, a multifunctional detection agent for concurrent use in computed tomography (CT) and optical imaging, or for concurrent use in positron emission tomography (PET) and optical imaging, also falls within the scope of this invention. Other diagnostic imaging techniques that may be combined with optical imaging include: X-ray based techniques, ultrasound, diagnostic techniques based on radioactive materials (*e.g.*, scintigraphy and SPECT), and the like.

The multifunctional contrast agents of the present invention can be formulated in any form, for example, a solid which is dissolved in a suitable carrier prior to use, or as a pre-made solution. When in the form of a solution, a wide range of concentrations is possible depending upon the desired dosing and method of introduction into tissue.

The carriers and adjunct ingredients which comprise the balance of the compositions of the present invention can be any pharmaceutically acceptable ingredient, for example, as a carrier distilled water. For embodiments wherein the contrast agent is provided as a solid which is reconstituted with water prior to use, the balance may comprise an inert filler. Or a suitable surfactant, anti-oxidant, or other stabilizer may be utilized.

The present invention further relates to a method for providing enhanced human and animal tissue differentiation by contrast imaging, wherein the multifunctional contrast agents of the present invention are taken up by tissue. The method of the present invention relates to establishing a blood serum level which is an effective amount of a contrast agent as described herein.

In one embodiment, the invention provides a method for providing to tissue a contrast agent thereby enabling differentiation of human or animal tissue, comprising the steps of: (a) administering to a human or non-human animal an effective amount of a multifunctional contrast agent which provides a contrast between tissues; and (b) sustaining said effective

amount of contrast agent for a period of time exceeding one hour. The serum levels for effective imaging will vary depending upon the uptake by the recipient, the type of tissue that is being targeted, and the lipophilicity of the contrast agent. The multifunctional contrast agent can be administered to the human or non-human animal in the form of a composition further comprising one or more carriers and, optionally, adjunct ingredients.

Administration of the multifunctional contrast agent of the present invention to a human or non-human animal subject, on whom imaging is to be performed, is achieved by conventional procedures known in by those of ordinary skill in the art and disclosed in the literature. Aqueous solutions of the agent are most conveniently used. The concentration of the agent in these solutions and the amounts administered may vary widely, the optimum in each case determined by the strength of the magnetic moment of the manganese atom, the contrast enhancement strength of the chelate as a whole and the method of administration, the degree of contrast enhancement desired or needed, and the age, weight, and condition of the subject to whom administration is made. Administration may be achieved by any route or method. For example, the contrast agent (and compositions comprising the contrast agent) can be administered parentally, such as by intravenous administration. One of skill in the art can readily determine appropriate dosages, concentrations, and rates and duration of administration, based on the size of the subject, the route of administration, and the imaging modality.

Imaging devices appropriate to the imaging modality can be utilized to obtain images of the desired anatomical information using the methods of the invention, and their selection can be readily determined by those skilled in the art. For example, the imaging device can be a magnetic resonance imaging device, a computed tomography device, a positron emission tomography device, or an optical imaging device, or combinations of two or more of the foregoing. If two or more imaging devices are used, they may be used simultaneously or consecutively.

In another embodiment, the multifunctional contrast agents of the invention can be associated with an implantable or deployable medical device or a pharmaceutically active agent, such as a drug, in order to detect and/or track the location of the device or pharmaceutically active agent *in vivo*.

In a preferred embodiment, the multifunctional contrast agents of the invention include targeting moieties. As used herein, the term "targeting moiety" is intended to mean a functional group that serves to target or direct the particle to a particular location or association (*e.g.*, a specific binding event). Thus, for example, a targeting moiety may be used to target a molecule to a specific target protein or enzyme, or to a particular cellular location, or to a particular cell type, to selectively enhance accumulation of the contrast agent. Suitable targeting moieties include, but are not limited to, polypeptides, nucleic acids, carbohydrates, lipids, hormones including proteinaceous and steroid hormones, growth factors, receptor ligands, antigens and antibodies, and the like. For example, as is more fully outlined below, the contrast agents of the invention may include a targeting moiety to target the agents to a specific cell type such as tumor cells, such as a transferrin moiety, since many tumor cells have significant transferrin receptors on their surfaces. Similarly, a targeting moiety may include components useful in targeting the contrast agents to a particular subcellular location. As will be appreciated by those in the art, the localization of proteins within a cell is a simple method for increasing effective concentration. For example, shuttling a drug into the nucleus confines them to a smaller space thereby increasing concentration. The physiological target may simply be localized to a specific compartment, and the agent must be localized appropriately. More than one targeting moiety can be conjugated or otherwise associated with each nanoparticle, and the target molecule for each targeting moiety can be the same or different.

Thus, suitable targeting sequences include, but are not limited to, binding sequences capable of causing binding of the moiety to a predetermined molecule or class of molecules, while retaining bioactivity of the expression product, (for example by using enzyme inhibitor or substrate sequences to target a class of relevant enzymes); sequences signaling selective degradation, of itself or co-bound proteins; and signal sequences capable of constitutively localizing the candidate expression products to a predetermined cellular locale, including (a) subcellular locations such as the Golgi, endoplasmic reticulum, nucleus, nucleoli, nuclear membrane, mitochondria, chloroplast, secretory vesicles, lysosome, and cellular membrane; and (b) extracellular locations via a secretory signal. Particularly preferred is localization to either subcellular locations.

The targeting moiety can function to target or direct the contrast agent to a particular location, cell type, diseased tissue, or association. In general, the targeting moiety is directed against a target molecule. As will be appreciated by those in the art, the contrast agents of the invention are generally injected intravenously; thus, preferred targeting moieties are those that allow concentration of the agents in a particular localization. Thus, for example, antibodies, cell surface receptor ligands and hormones, lipids, sugars and dextrans, alcohols, bile acids, fatty acids, amino acids, peptides and nucleic acids may all be attached to localize or target the contrast agent to a particular site.

In preferred embodiments, the targeting moiety allows targeting of the contrast agents of the invention to a particular tissue or the surface of a cell. That is, in a preferred embodiment, the contrast agents of the invention need not be taken up into the cytoplasm of a cell to be activated.

In some embodiments, the targeting moiety is a peptide. For example, chemotactic peptides have been used to image tissue injury and inflammation, particularly by bacterial infection; see WO 97/14443, hereby expressly incorporated by reference in its entirety.

In some embodiments, the targeting moiety is an antibody. The term "antibody" includes antibody fragments, as are known in the art, including Fab or Fab_2, single chain antibodies (Fv for example), chimeric antibodies, *etc.*, either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA technologies.

In a preferred embodiment, the antibody targeting moieties of the invention are humanized antibodies or human antibodies. Humanized forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which

are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992)).

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-327 (1988); Verhoeyen *et al.*, *Science* 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries (Hoogenboom and Winter, J. Mol. Biol. 227:381 (1991); Marks et al., J. Mol. Biol. 222:581 (1991)). The techniques of Cole *et al.* and Boerner *et al.* are also available for the preparation of human monoclonal antibodies (Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985) and Boerner *et al.*, *J. Immunol.* 147(1):86-95 (1991)). Similarly, human antibodies can be made by introducing human immunoglobulin loci into transgenic animals, *e.g.*, mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects,

including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks *et al.*, *Bio/Technology* 10:779-783 (1992); Lonberg *et al.*, *Nature* 368:856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild *et al.*, *Nature Biotechnology* 14:845-51 (1996); Neuberger, *Nature Biotechnology*, 14:826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995).

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for a first target molecule and the other one is for a second target molecule.

Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism (see, for example, Suresh *et al.*, *Methods in Enzymology* 121:210 (1986)).

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Patent No. 4,676,980.

In a preferred embodiment, the antibody is directed against a cell-surface marker on a cancer cell; that is, the target molecule is a cell surface molecule. As is known in the art, there

are a wide variety of antibodies known to be differentially expressed on tumor cells, including, but not limited to, HER2, VEGF, *etc.*

In addition, antibodies against physiologically relevant carbohydrates may be used, including, but not limited to, antibodies against markers for breast cancer (CA15-3, CA 549, CA 27.29), mucin-like carcinoma associated antigen (MCA), ovarian cancer (CA125), pancreatic cancer (DE-PAN-2), and colorectal and pancreatic cancer (CA 19, CA 50, CA242).

In one embodiment, antibodies against virus or bacteria can be used as targeting moieties. As will be appreciated by those in the art, antibodies to any number of viruses (including orthomyxoviruses, (*e.g.*, influenza virus), paramyxoviruses (*e.g.*, respiratory syncytial virus, mumps virus, measles virus), adenoviruses, rhinoviruses, coronaviruses, reoviruses, togaviruses (*e.g.*, rubella virus), parvoviruses, poxyiruses (*e.g.*, variola virus, vaccinia virus), enteroviruses (*e.g.*, poliovirus, coxsackievirus), hepatitis viruses (including A, B and C), herpesviruses (*e.g.*, Herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus), rotaviruses, Norwalk viruses, hantavirus, arenavirus, rhabdovirus (*e.g.*, rabies virus), retroviruses (including HIV, HTLV-I and -II), papovaviruses (*e.g.*, papillomavirus), polyomaviruses, and picornaviruses, and the like), and bacteria (including a wide variety of pathogenic and non-pathogenic prokaryotes of interest including Bacillus; Vibrio, *e.g.*, V. cholerae; Escherichia, *e.g.*, Enterotoxigenic E. coli, Shigella, *e.g.*, S. dysenteriae; Salmonella, *e.g.*, S. typhi; Mycobacterium *e.g.*, M. tuberculosis, M. leprae; Clostridium, *e.g.*, C. botulinum, C. tetani, C. difficile, C.peffringens; Cornyebacterium, *e.g.*, C. diphtheriae; Streptococcus, S. pyogenes, S. pneumoniae; Staphylococcus, *e.g.*, S. aureus; Haemophilus, *e.g.*, H. influenzae; Neisseria, *e.g.*, N. meningitidis, N. gonorrhoeae; Yersinia, *e.g.*, G. lamblia Y. pestis, Pseudomonas, *e.g.*, P. aeruginosa, P. putida; Chlamydia, *e.g.*, C. trachomatis; Bordetella, *e.g.*, B. pertussis; Treponema, *e.g.*, T. palladium; and the like) may be used.

In a preferred embodiment, the targeting moiety is all or a portion (*e.g.*, a binding portion) of a ligand for a cell surface receptor. Suitable ligands include, but are not limited to, all or a functional portion of the ligands that bind to a cell surface receptor selected from the group consisting of insulin receptor (insulin), insulin-like growth factor receptor (including both IGF-1 and IGF-2), growth hormone receptor, glucose transporters (particularly GLUT 4 receptor),

transferrin receptor (transferrin), epidermal growth factor receptor (EGF), low density lipoprotein receptor, high density lipoprotein receptor, leptin receptor, estrogen receptor (estrogen); interleukin receptors including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12, IL-13, IL-15, and IL-17 receptors, human growth hormone receptor, VEGF receptor (VEGF), PDGF receptor (PDGF), transforming growth factor receptor (including TGF-.alpha. and TGF-.beta.), EPO receptor (EPO), TPO receptor (TPO), ciliary neurotrophic factor receptor, prolactin receptor, and T-cell receptors. In particular, hormone ligands are preferred. Hormones include both steroid hormones and proteinaceous hormones, including, but not limited to, epinephrine, thyroxine, oxytocin, insulin, thyroid-stimulating hormone, calcitonin, chorionic gonadotropin, cortictropin, follicle-stimulating hormone, glucagon, leuteinizing hormone, lipotropin, melanocyte-stimutating hormone, norepinephrine, parathryroid hormone, thyroid-stimulating hormone (TSH), vasopressin, enkephalins, seratonin, estradiol, progesterone, testosterone, cortisone, and glucocorticoids and the hormones listed above. Receptor ligands include ligands that bind to receptors such as cell surface receptors, which include hormones, lipids, proteins, glycoproteins, signal transducers, growth factors, cytokines, and others.

In another embodiment, the targeting moiety is a carbohydrate. As used herein, the term "carbohydrate" includes compounds with the general formula $Cx(H_2O)_y$. Monosaccharides, disaccharides, and oligo- or polysaccharides are all included within the definition and comprise polymers of various sugar molecules linked via glycosidic linkages. Particularly preferred carbohydrates are those that comprise all or part of the carbohydrate component of glycosylated proteins, including monomers and oligomers of galactose, mannose, fucose, galactosamine, (particularly N-acetylglucosamine), glucosamine, glucose and sialic acid, and in particular the glycosylation component that allows binding to certain receptors such as cell surface receptors. Other carbohydrates comprise monomers and polymers of glucose, ribose, lactose, raffinose, fructose, and other biologically significant carbohydrates. In particular, polysaccharides (including, but not limited to, arabinogalactan, gum arabic, mannan, *etc.*) have been used to deliver MRI agents into cells; *see* U.S. Patent No. 5,554,386, hereby incorporated by reference in its entirety.

In another embodiment, the targeting moiety is a lipid. As used herein, the term "lipid" includes fats, fatty oils, waxes, phospholipids, glycolipids, terpenes, fatty acids, and glycerides, particularly the triglycerides. Also included within the definition of lipids are the eicosanoids, steroids and sterols, some of which are also hormones, such as prostaglandins, opiates, and cholesterol.

In a preferred embodiment, the targeting moiety may be used to either allow the internalization of the contrast agent to the cell cytoplasm or localize it to a particular cellular compartment, such as the nucleus. In a preferred embodiment, the targeting moiety is all or a portion of the HIV-1 Tat protein, and analogs and related proteins, which allows very high uptake into target cells (See for example, Fawell *et al.*, *PNAS USA* 91:664 (1994); Frankel *et al.*, *Cell* 55:1189 (1988); Savion *et al.*, *J. Biol. Chem.* 256:1149 (1981); Derossi *et al.*, *J. Biol. Chem.* 269:10444 (1994); and Baldin *et al.*, *EMBO J.* 9:1511 (1990), all of which are incorporated by reference.

In a preferred embodiment, the targeting moiety is a nuclear localization signal (NLS). NLSs are generally short, positively charged (basic) domains that serve to direct the moiety to which they are attached to the cell's nucleus. Numerous NLS amino acid sequences have been reported including single basic NLS's such as that of the SV40 (monkey virus) large T Antigen (Pro Lys Lys Lys Arg Lys Val), Kalderon (1984), *et al.*, *Cell*, 39:499-509; the human retinoic acid receptor-.beta. nuclear localization signal (ARRRRP); NFκB p50 (EEVQRKRQKL; Ghosh *et al.*, *Cell* 62:1019 (1990); NF.kappa.B p65 (EEKRKRTYE; Nolan *et al.*, Cell 64:961 (1991); and others (see for example Boulikas, *J. Cell. Biochem.* 55(1):32-58 (1994), hereby incorporated by reference) and double basic NLS's exemplified by that of the *Xenopus* (African clawed toad) protein, nucleoplasmin (Ala Val Lys Arg Pro Ala Ala Thr Lys Lys Ala Gly Gln Ala Lys Lys Lys Lys Leu Asp), Dingwall, *et al.*, Cell, 30:449-458, 1982 and Dingwall, *et al.*, *J. Cell Biol.*, 107:641-849; 1988). Numerous localization studies have demonstrated that NLSs incorporated in synthetic peptides or grafted onto reporter proteins not normally targeted to the cell nucleus cause these peptides and reporter proteins to be concentrated in the nucleus (see, for example, Dingwall, and Laskey, *Ann, Rev. Cell Biol.*, 2:367-390, 1986; Bonnerot, *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:6795-6799, 1987; Galileo, *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:458-462, 1990.

In another embodiment, targeting moieties for the hepatobiliary system are used (see U.S. Patent Nos. 5,573,752 and 5,582,814, both of which are hereby incorporated by reference in their entirety).

In specific embodiments, a cell-binding agent is utilized as the targeting moiety. Selection of the appropriate cell-binding agent is a matter of choice that depends upon the particular cell population to be targeted, but in general monoclonal antibodies are preferred if an appropriate one is available.

For example, the monoclonal antibody MY9 is a murine IgG_1 antibody that binds specifically to the CD33 antigen (J. D. Griffin *et al. Leukemia Res.*, 8: 521 (1984)) which can be used if the target cells express CD33, such as in the disease of acute myelogenous leukemia (AML). Similarly, the monoclonal antibody anti-B4 is a murine IgG_1 that binds to the CD19 antigen on B cells (Nadler *et al., J. Immunol.* 131: 244-250 (1983)) and can be used if the target cells are B cells or diseased cells that express this antigen, such as in non-Hodgkin's lymphoma or chronic lymphoblastic leukemia. Similarly, the antibody N901 is a murine monoclonal IgG_1 antibody that binds to CD56 found on small cell lung carcinoma cells and on cells of other tumors of neuroendocrine origin (Roy *et al. J. Nat. Cancer Inst.* 88:1136-1145 (1996)).

Antibodies that target solid tumors are also useful, such as the C242 antibody which binds to a carbohydrate antigen found on MUC1 present on pancreatic and colorectal tumors. (U.S. Patent No. 5,552,293); antibody J591, which binds to PSMA (prostate specific membrane antigen) which is expressed on prostate cancer cells and on endothelial cells of neovasculature in tumors (U.S. Patent No. 6,107,090, He Liu *et al.* Cancer Res. 57: 3629-3634 (1997); and antibodies to HER-2, which is overexpressed on certain breast tumors. Examples of anti-HER-2 antibodies are the TA1 antibody (L. A. Maier *et al. Cancer Res.* 51: 5361-5369 (1991)) and the 4D5 antibody (U.S. Patent Nos. 6,387,371 and 6,399,063).

Additionally, GM-CSF, which binds to myeloid cells, can be used as a cell-binding agent to diseased cells from acute myelogenous leukemia. IL-2, which binds to activated T-cells, can be used for prevention of transplant graft rejection, for therapy and prevention of graft-versus-host disease, and for treatment of acute T-cell leukemia. MSH, which binds to melanocytes can

be used for the treatment of melanoma. Folic acid, which targets the folate receptor expressed on ovarian and other cancers, is also a suitable cell-binding agent.

Cancers of the breast and testes can be successfully targeted with estrogen (or estrogen analogues) or androgen (or androgen analogues), respectively, as cell-binding

As is further described below, the contrast agents of the present invention find use in a wide variety of applications. These applications include *in vitro* and *in vivo* monitoring of gene expression, disease progression, drug response(s) and biodistribution assays. In particular, *in vivo* real time acquisition of data in living animals that are not sacrificed can be a powerful tool. In addition, *in vitro* and *in vivo* imaging of cells, tissues and animals may be done using a plurality of these agents, particularly utilizing sequential administration and image collection.

The contrast agents of the invention may be used in a similar manner to the known gadolinium MRI agents (see for example, Meyer *et al.*, *supra*; U.S. Patent No. 5,155,215; U.S. Pat. No. 5,087,440; Margerstadt *et al.*, *Magn. Reson. Med.* 3:808 (1986); Runge *et al.*, *Radiology* 166:835 (1988); and Bousquet *et al., Radiology* 166:693 (1988). The metal ion complexes are administered to a cell, tissue or patient as is known in the art.

The terms "patient", "recipient", "subject", and "host" are used interchangeably and, for the purposes of the present invention, include both humans and other animals and organisms, such as experimental animals. Thus, the methods are applicable to both human therapy and veterinary applications, as well as research. In addition, the contrast agents of the invention may be used to image tissues or cells (for example, see Aguayo *et al.*, *Nature* 322:190 (1986)).

Mammalian species which benefit from the methods and contrast agents of the invention include, and are not limited to, apes, chimpanzees, orangutans, humans, monkeys; domesticated animals (*e.g.*, pets) such as dogs, cats, guinea pigs, hamsters, Vietnamese pot-bellied pigs, rabbits, and ferrets; domesticated farm animals such as cows, buffalo, bison, horses, donkey, swine, sheep, and goats; exotic animals typically found in zoos, such as bear, lions, tigers, panthers, elephants, hippopotamus, rhinoceros, giraffes, antelopes, sloth, gazelles, zebras, wildebeests, prairie dogs, koala bears, kangaroo, opossums, raccoons, pandas, hyena, seals, sea lions, elephant seals, otters, porpoises, dolphins, and whales. Thus, as used herein, the terms

"patient", "recipient", "subject", and "host" are intended to include such human and non-human species.

The terms "comprising", "consisting of" and "consisting essentially of" are defined according to their standard meaning. The terms may be substituted for one another throughout the instant application in order to attach the specific meaning associated with each term. The phrases "isolated" or "biologically pure" refer to material that is substantially or essentially free from components which normally accompany the material as it is found in its native state.

The terms "linked", "joined", "grafted", "tethered", "associated", and "conjugated" in the context of the nanoparticles of the invention, are used interchangeably to refer to any method known in the art for functionally connecting moieties (such as targeting moieties), including, without limitation, recombinant fusion, covalent bonding, disulfide bonding, ionic bonding, hydrogen bonding, and electrostatic bonding.

As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a nanoparticle" includes more than one such nanoparticle, and the like. Reference to "a targeting moiety" includes more than one such targeting moiety. Reference to an "image" includes more than one such image. For example, an image can include one or more "stills" or "screen shots", or a stream of continuous images and recorded as a video.

The practice of the present invention can employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology, electrophysiology, and pharmacology, that are within the skill of the art. Such techniques are explained fully in the literature (see, *e.g.*, Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989); DNA Cloning, Vols. I and II (D. N. Glover ed. 1985); Perbal, B., A Practical Guide to Molecular Cloning (1984); the series, Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Transcription and Translation (Hames *et al.* eds. 1984); Gene Transfer Vectors For Mammalian Cells (J. H. Miller *et al.* eds. (1987) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); Scopes, Protein Purification: Principles and Practice (2nd ed., Springer-Verlag); and PCR: A Practical Approach (McPherson *et al.* eds. (1991) IRL Press)).

Following are examples that illustrate materials, methods, and procedures for practicing the invention. The examples are illustrative and should not be construed as limiting.

Materials and Methods

Synthesis of CdS:Mn/ZnS Core/Shell Quantum Dots. Yellow-emitting Mn^{2+} doped core/shell nanocrystals of ZnS-passivated CdS:Mn were produced via a reverse micelle process. (Cd^{2+} + Mn^{2+})-containing aqueous solution is prepared by dissolving $Cd(CH_3COO)_2 \cdot 2H_2O$ and $Mn(CH_3COO)_2$ in water, and S^{2-}- and Zn^{2+}-containing aqueous solutions are prepared respectively by dissolving Na_2S and $Zn(CH_3COO)_2$ in water. Each solution was stirred with an anionic surfactant AOT/heptane stock solution. CdS:Mn/ZnS quantum dots are formed by mixing (Cd^{2+} + Mn^{2+})- and S^{2-}-containing micellar solutions for 15 min, followed by the addition of Zn^{2+}-containing micellar solution at a very slow rate of ~1.5 ml/min for ZnS shell growth over the CdS:Mn core surface. A surplus of S ions was maintained in the CdS:Mn nanocrystal micellar solution for further ZnS shell growth. All reactions were conducted at room temperature. The concentrations of Cd^{2+}, S^{2-}, and Zn^{2+} in water were 0.1, 0.66, and 0.26 M, respectively (0.048 g of $Cd(CH_3COO)_2 \cdot 2H_2O$ in 1.8 ml of water, 0.2812 g of Na_2S in 5.4 ml of water, and 0.264 g of $Zn(CH_3COO)_2$ in 5.4 ml of water are used). The Mn solution concentration in CdS is 2 mol % (0.00062 g of $Mn(CH_3COO)_2$ is used). The molar ratio of water-to-surfactant (*W*) is 10 for all micellar solutions. The concentration of AOT in heptane is 0.1 M. More detailed compositions of each micellar solution are shown in Table 1.

Table 1. Compositions of Each Micellar Solution

	(Cd^{2+} + Mn^{2+})- micellar solution	S^{2-}- micellar solution	Zn^{2+}- micellar solution
Water	1.8 ml	5.4 ml	5.4 ml
AOT	4.46 g	13.38 g	13.38 g
Heptane	100 ml	300 ml	300 ml

Silica coating and Subsequent Surface-Functionalization of Quantum Dots. After addition of the Zn micellar solution, 2.5 ml of tetraethyl orthosilicate (TEOS) is injected into CdS:Mn/ZnS micellar solution and mixed for 15 min at room temperature. The hydrolysis of TEOS and condensation reaction is initiated by adding NH_4OH in the form of micellar solution, which is prepared by mixing 1.5 ml of NH_4OH (30 wt %) with AOT (3.69 g)/heptane (82.5 ml) stock solution. After polymerization for 24 hr at room temperature, 1.25 ml of TEOS and 0.25 ml of 3-(Aminopropyl)triethoxysilane (APTS) are injected into above solution and mixed for 10 min. And then, another NH_4OH micellar solution (prepared by mixing 0.9 ml of NH_4OH with AOT (2.21 g)/heptane (50 ml) stock solution) and 3-(Trihydroxysilyl)propyl methylphosphonate (THPMP) (prepared by mixing 0.75 ml of THPMP and 3.6 ml of water with AOT (4.42 g)/heptane (50 ml) stock solution) are injected subsequently and reacted for 24 hr. And silica-overcoated-, surface-functionalized quantum dots are precipitated by addition of a small amount of methanol. After thorough washing with methanol, these quantum dots are solubilized stably in a sodium phosphate buffer solution (pH ~7).

Characterization. A JEOL JSM 6400 electron microscope operated at 15 kV was used for EDS analysis. A Perkin-Elmer PHI 5100 x-ray photoelectron spectrometer and Mg K_α x-ray (1253.6 eV) were used for XPS/ESCA. Survey scans were collected from 1100 to 0 eV with a step of 0.5 eV, a time/step of 30 ms, and a pass energy of 89.45 eV. Multiplex scans were colleted with a step of 0.1 eV, a time/step of 50 ms, and pass energy of 35.75 eV. A JEOL 2010F transmission electron microscope operated at 200 kV was used for imaging and direct determination of the nanocrystal size. The XRD patterns were obtained with a Philips MRD X'Pert system for information on structure and crystal size. XRD pattern was collected in the step scan mode, typically with a scan range of $15^\circ - 80^\circ$, a step of 0.01°, and a glazing angle of incident x-ray of 1°.

Quantum yields of *n*-dodecanethiol- and ZnS-passivated CdS:Mn nanocrystals dispersed in chloroform were determined by comparing the integrated emission to those from Coumarin 6, Coumarin 30, and perylene at the excitation wavelength of 385 nm. Coumarin 6, Coumarin 30,

and perylene were dissolved in ethanol, acetonitrile, and cyclohexane, respectively. The optical densities of the sample and reference solutions were 0.065 ± 0.003 at the excitation wavelength.

Example 1—Core/Shell Structure and Size of Nanocrystals

It has been reported that synthesized nanocrystals consist of a CdS core/ZnS shell structure, based on UV-visible absorption spectroscopy and x-ray photoelectron spectroscopy (XPS)/energy-dispersive spectroscopy (EDS) (Yang, H. and P. H. Holloway, *Appl. Phys. Lett.* 2003, 82:1965-1967). Since XPS is a surface sensitive method and EDS is a 'bulk' method of analysis (Brundle, C.R., Encyclopedia of Materials Characterization, (Eds: C. R. Brundle, C. A. Evans, Jr., S. Wilson), Butterworth-Heinemann, Boston 1992), the samples with CdS core/ZnS shell structure should experience an attenuation of the signals from buried core element, *i.e.*, Cd, in XPS relative to EDS. Thus XPS spectra should be dominated by the shell, while EDS spectra should exhibit the elements in both the shell and the core structure averaged over many nanocrystal thickness. XPS survey spectra of CdS:Mn, ZnS:Mn, and CdS:Mn/ZnS core/shell nanocrystals are shown in Figure1. The primary Cd $3d_5$ and Zn $2p_3$ XPS lines are observed in Figure 1, trace (a) and trace (b), respectively, and those two lines are present in trace (c) of Figure 1. Accurate quantitative compositions of Zn/Cd in XPS were obtained by integrating the peak area and dividing by the atomic sensitivity factors (Wagner, C.D., W. M. Riggs, L. E. Davis, J. F. Moulder, G. E. Mullenberg, Handbook of X-ray Photoelectron Spectroscopy, Perkin Elmer Corp., Eden Prarie, MN 1979). The Zn/Cd ratios from EDS and XPS in CdS:Mn/ZnS nanocrystals are 5.5 and 6.6, respectively, supporting that the nanocrystals consist of layered structure. Transmission electron microscope (TEM) images of CdS:Mn/ZnS core/shell nanocrystals, whose average size is observed to be 3.1 nm, are shown in Figure 2A. The size of the nanocrystal core was measured using XRD patterns (Figure 2B) reduced with the Debye-Scherrer equation and found to be 2.3 nm. Since the shell layer does not affect the X-ray diffraction (XRD) peak width from the core (X. Peng, M. C. Schlamp, A. V. Kadavanich, A. P. Alivisatos, *J. Am. Chem. Soc.* 1997, 119, 7019. L. Manna, E. C. Scher, L. Li, A. P. Alivisatos, *J. Am. Chem. Soc.* 2002, 124:7136), the difference between the TEM and XRD diameters (0.4 nm) can be attributed to the ZnS shell thickness (~1.5 monolayers of ZnS). Diffraction peaks from

nanocrystals matched those of bulk zinc blende CdS (Joint Committee on Powder Diffraction Standards (JCPDS), International Center for Diffraction Data (ICDD), Swarthmore, PA). The inset in Figure 2A shows atom resolution images of a nanocrystal with the zinc blende structure and the $(1\bar{1}0)$ and (110) planes are identified.

Example 2—Photoluminescence (PL) and Quantum Yield

PL emission spectra, obtained using 325 nm HeCd laser excitation, of *n*-dodecanethiol-passivated and ZnS-passivated CdS:Mn nanocrystals are compared in Figure 3A and Figure 3B, respectively. Note that PL measurements were carried out in the solid-state sample, *i.e.*, ~200 nm thick CdS:Mn/ZnS nanocrystal layers on the glass substrate. The Mn^{2+} $^4T_1$-$^6A_1$ transition at ~600 nm is observed from both nanocrystalline samples. In addition to the Mn emission at ~600 nm, emission from a surface-related defect (shallow trap) is observed at ~450 nm from organically passivated nanocrystals. This defect emission originates from the localized surface states in the band gap, which presumably are formed by the lack of bonding to surface S ions. In contrast, no defect-related emission is observed from inorganically passivated nanocrystals, indicating the successful complete passivation of CdS:Mn core surface by the ZnS shell layer.

The quantum yields of CdS:Mn nanocrystals with either a *n*-dodecanethiol or ZnS capping layer were measured in chloroform solutions and found to be 3.7 and 18 %, respectively. Note that for quantum yield measurement, CdS:Mn/ZnS core/shell nanocrystals were capped with *n*-dodecanethiol in order to be soluble in chloroform. The value of 3.7 % for organically capped CdS:Mn is reasonable since it is close to the quantum yields reported from organically passivated ZnS:Mn nanocrystals (1-4 %) (Kubo, T., T. Isobe, M. Senna, *J. Lumin.* 2002, 99:39; Bol, A., A. Meijerink, *J. Phys. Chem. B* 2001, *105*, 10197). The enhanced quantum yield of *n*-dodecanethiol-capped CdS:Mn/ZnS nanocrystals is a direct result of more effective surface passivation, by which nonradiative recombination paths are reduced significantly. A visual comparison of organically and inorganically passivated nanocrystals is shown in Figure 4. Both samples are standing on a handheld-UV lamp providing 366 nm multiband irradiation, and it is obvious that the ZnS-passivated nanocrystals are much brighter.

Example 3—Effects of UV irradiation and Photooxidation

The change of PL emission intensity from organically and inorganically passivated CdS:Mn during 400 nm UV irradiation was monitored at room temperature using a monochromatized 300 W Xe light source (Figure 5). The 400 nm UV irradiation was estimated to have a powder density of 655 $\mu W/cm^2$. Organically passivated CdS:Mn nanocrystals exhibit a significant reduction (~45 % after 3 hr) of the PL emission intensity upon UV exposure, while CdS:Mn/ZnS core/shell nanocrystals show an increased PL intensity (~40 % after 3 hr). The reduced PL emission intensity of *n*-dodecanethiol-passivated CdS:Mn nanocrystals presumably results from the fact that the bonding between the Cd (from nanocrystal) and S ions (from organic passivating species) at the surface is deteriorated by UV exposure. Accordingly, the density of nonradiative relaxation paths increases at the surface, resulting in a decreased PL emission intensity (C. Jin, C., J. Yu, L. Sun, K. Dou, S. Hou, U. Zhao, Y. Chen, S. Huang, *J. Lumin.* 1996, 66-67, 315; Pingbo, X., Z. Weiping, Y. Min, C. Houtong, Z. Weiwei, L. Liren, X. Shangda, *J. Colloid Interface Sci.* 2000, *229*, 534). The increased PL emission intensity observed in CdS:Mn/ZnS nanocrystals indicates the creation of more radiative or reduction of non-radiative paths as a result of UV irradiation. Enhancement of PL quantum efficiency of unpassivated ZnS:Mn nanocrystal as a result of UV exposure has been reported by other research groups (Bol, A., A. Meijerink, *J. Phys. Chem. B* 2001, 105:10203; Cao, L., J. Zhang, S. Ren, S. Huang, *Appl. Phys. Lett.* 2002, *80*, 4300). Bol and Meijerink (2001) reported that a photochemical reaction (photooxidation) of oxygen and/or water with the surface of ZnS nanocrystal surface occurs, leading to the formation of $ZnSO_4$ and/or $Zn(OH)_2$, and these phases serve as passivating layers on the ZnS surface to reduce the nonradiative recombination paths. To study this possibility, XPS data were collected in the current study and are shown in Figure 6. The S 2p from CdS:Mn/ZnS nanocrystals irradiated with UV for 3 hr in either air or Ar atmospheres are shown. This UV irradiation treatment was accomplished using a handheld UV lamp providing 366 nm multiband photons with a power density of 1350 $\mu W/cm^2$. The S 2p peaks at ~166.5 and ~173.8 eV are due to S atoms in the CdS core/ZnS shell and in $ZnSO_4$, respectively (National Institute of Standards and Technology (NIST), X-ray Photoelectron Spectroscopy Database 20, Ver. 3.3). Clearly, $ZnSO_4$ is only formed by irradiation in air

supporting the suggestion by Bol *et al.* (2001) that photooxidation occurs between the ZnS shell surface and oxygen or water in air but not in Ar. The small peak at ~173.8 eV for irradiation in Ar is the result of exposure to air prior to UV irradiation. These data support the conclusion that photooxidation production of a passivating surface sulfate is responsible for the enhanced PL emission. The PL intensity after 3 hr UV irradiation from core/shell nanocrystals remained constant when the UV irradiation was stopped for periods up to 2 hr and restarted. This observation would imply that the $ZnSO_4$ is stable and the photooxidation process is irreversible.

The variation with 350 nm UV irradiation time of the PL emission intensity from *n*-dodecanethiol-capped CdS:Mn/ZnS nanocrystals is shown Fig. 7. A 60 % increased brightness was observed for an exposure of 3 hr, i.e., slightly greater than the increase reported above (40 %) for a 3 hr exposure of CdS:Mn/ZnS nanocrystals (no *n*-dodecanethiol capping) to 400 nm UV. While the quantum yield of *n*-dodecanethiol-capped CdS:Mn/ZnS nanocrystals was not measured after the PL intensity was increased by UV irradiation for 3 hr, it may be estimated by multiplying the quantum yield without UV enhancement (18 %) by the enhanced brightness (60 %). Using this procedure, a final quantum yield of >28 % would be expected.

Thus, synthesis of efficient and photostable ZnS-passivated CdS:Mn core/shell nanocrystals via a reverse micelle route has been described herein. Nanocrystals with a CdS:Mn/ZnS core/shell structure with a core crystal diameter of 2.3 nm and a shell thickness of 0.4 nm were obtained. Effective passivation of the CdS:Mn core surface by a ZnS shell was evident from the absence of a surface-related defect emission and a high quantum efficiency (18 %) compared to *n*-dodecanethiol-passivated CdS:Mn nanocrystals (surface defect luminescence observed and a quantum yield of only 3.7 %). Although *n*-dodecanethiol-passivated CdS:Mn nanocrystals suffered from the reduction of emission intensity during UV irradiation, ZnS-passivated core/shell nanocrystals exhibited the enhanced emission intensity during initial UV irradiation and saturated, stable emission during further UV irradiation. XPS data supported that CdS:Mn/ZnS nanocrystals experience the photooxidation during UV irradiation in air atmosphere. The surface sulfate ($ZnSO_4$), formed as a result of photooxidation, serves as a passivating layer and is responsible for the enhanced PL emission. Also it was concluded that

the $ZnSO_4$ is stable and the photooxidation process is irreversible. An even higher quantum yield (>28 %) was estimated from these UV irradiated core/shell nanocrystals.

Example 4—Magnetic Properties of CdS:Mn/ZnS Core/Shell Quantum Dots

The magnetic properties of the quantum dots were studied by a SQUID magnetometer. About 10 mg of the quantum dots in powder form was inserted in a gelatin capsule for the magnetic measurement. The temperature-dependent applied field versus magnetization curves of Mn-doped and undoped core/shell quantum dots are shown in Figures 7A-7C and Figures 8A and 8B, respectively. As shown in Figures 7A-7C, Mn-doped core/shell quantum dots exhibit distinct transit with temperature from linear behavior (Figure 7A) to hysterisis (Figure 7C), indicative of their ferromagnetism at 300 K. However, undoped quantum dots show weak temperature-dependent magnetic behavior. Also note that there is a relatively large difference in magnetic responses (magnetization) of two types of quantum dots, *i.e.*, 1.2×10^{-5} (emu) of Mn-doped quantum dots versus 6×10^{-6} (emu) of undoped counterparts at 1000 Oe of magnetic field measured at 300 K.

Example 5—Synthesis of TAT-Conjugated Quantum Dots

For 2-pyridyl disulfide derivatized quantum dots, to 7 ml of silica-overcoated, surface-functionalized quantum dot solution, with approximate particle concentration of 10^{16}/ml, is added 25 mg of *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) in 0.5 ml of dimethy sulfoxide (DMSO). The mixture is allowed to react for 12 hours at room temperature. Low molecular byproducts are removed via a gel permeation chromatography (GPC), equilibrated with 0.01 M tris base and 0.02 M citric acid, pH 7.4 buffer. To 7 ml of 2-pyridyl disulfide derivatized quantum dots is added 4 mg of TAT peptide (TAT peptide sequence: <u>Gyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg</u>-Gly-Tyr-Cys-NH_2; 10 amino acid active component of TAT peptide is underlined) in 0.2 ml of DMSO. The mixture is allowed to react for 12 hours at room temperature. The TAT-conjugated quantum dots are centrifuged (8000 rpm, 8 min) and dispersed in DMSO.

Example 6—Bioimaging Using TAT-Conjugated Quantum Dots

Rats (250-300g) were anesthetized with a Ketamine/Xylazine mixture (0.1ml/100g) administered intraperitonealy (IP). The necks were shaved and prepped with chlorhexidine scrub. A 2-3cm long incision was made on the neck. The right common carotid (RCC) artery was located and isolated from the surrounding tissue. 4-0 silk suture was used to ligate the common carotid proximally. Another length of suture was wrapped around the external carotid and occipital arteries to temporarily stop blood flow during injection of the substance. A small sheath was placed in the RCC and secured in place. The experimental substance (0.75ml of TAT-grafted quantum dots (CdS:MN/ZnS) at a concentration of 10 mg/ml) was loaded into a syringe and placed into an infusion pump. The syringe was attached to the sheath and the pump was started. The infusion pump injected the substance over a period of five minutes. After the injection of the substance, the external carotid artery was opened and collateral blood flow allowed for 3 minutes. The rat was then euthanized with an overdose of sodium pentobarbital. A craniotomy was performed on the rat after euthanasia and the brain was removed. The brain was analyzed grossly and a handheld ultraviolet (UV) source was used to confirm staining of the brain by the substance. The brain was then sliced into (4) 5mm sections and placed into 10% buffered formalin. The tissue samples were processed, embedded in paraffin, and 2 unstained slides were made from each section of brain. The slides were analyzed under a fluorescence microscope. Results are shown in Figures 10A-B, 11, and 12.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

Abstract

The present invention relates to fluorescent, radio-opaque and magnetic quantum nanoparticles, useful as multifunctional contrast agents or probes for *in vivo* bioimaging, and methods of their use. The invention provides for multifaceted bioimaging (*e.g.*, intra-arterial pre-operative brain mapping and broad based *in vivo* diagnostic imaging).

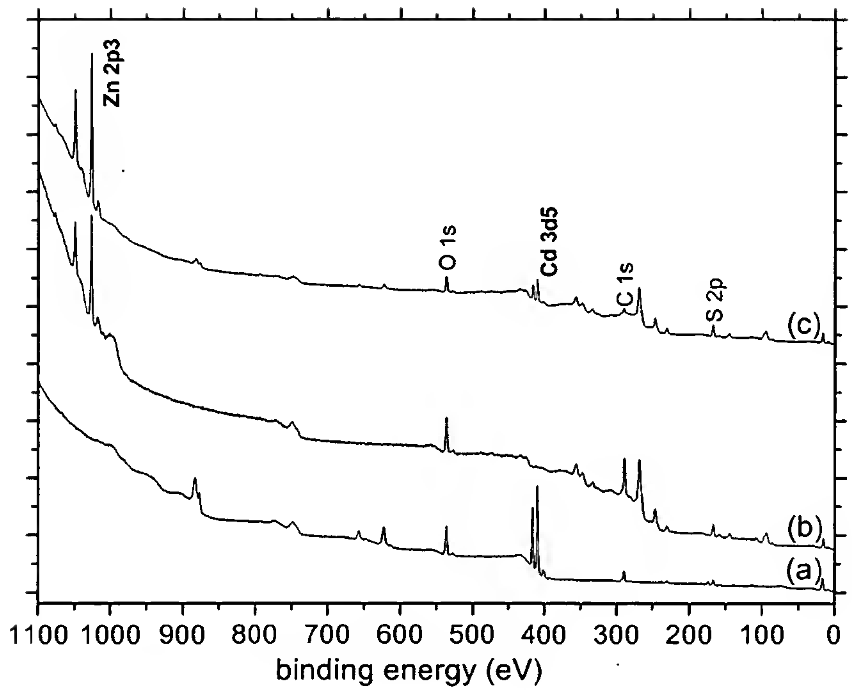

Zn 2p3
O 1s
Cd 3d5
C 1s
S 2p
(c)
(b)
(a)
1100 1000 900 800 700 600 500 400 300 200 100 0
binding energy (eV)

FIG. 1

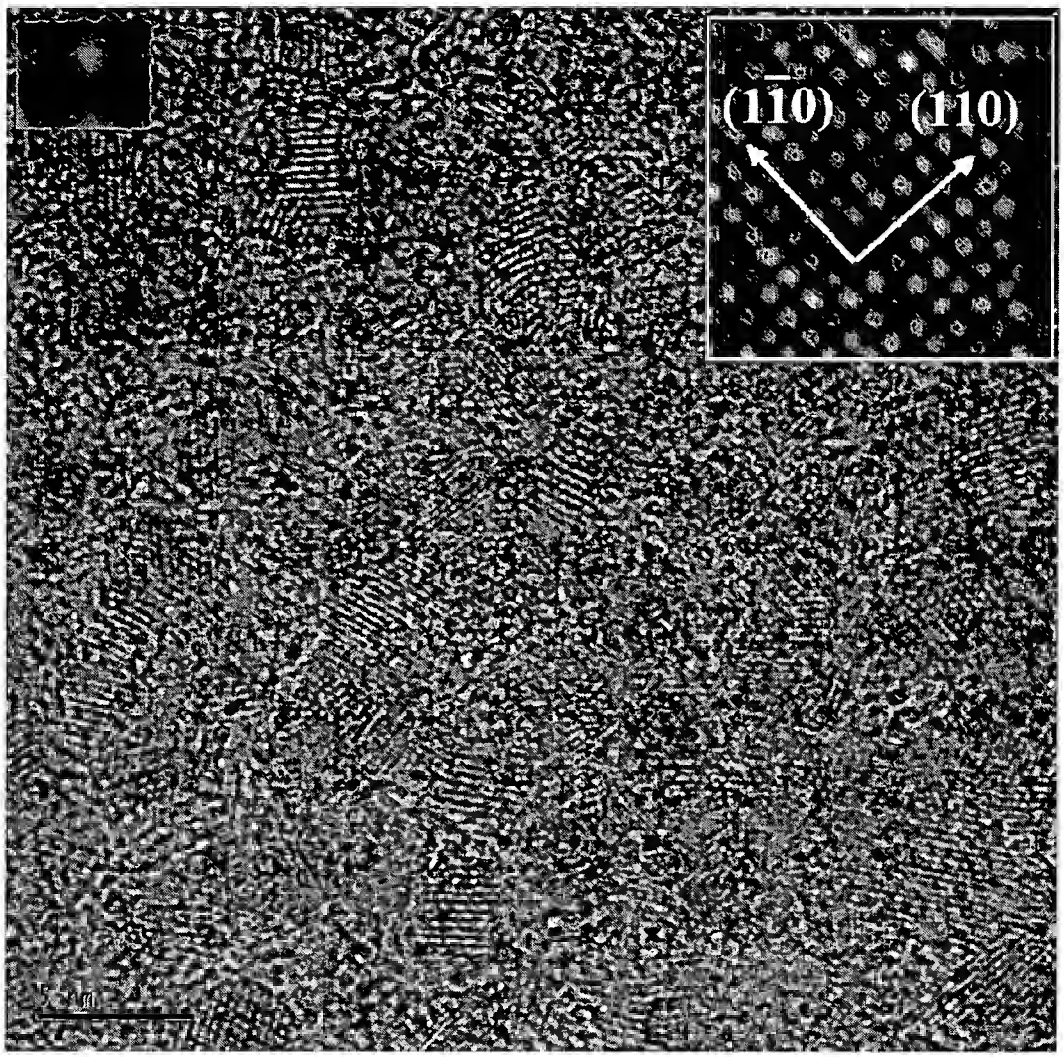


FIG. 2A

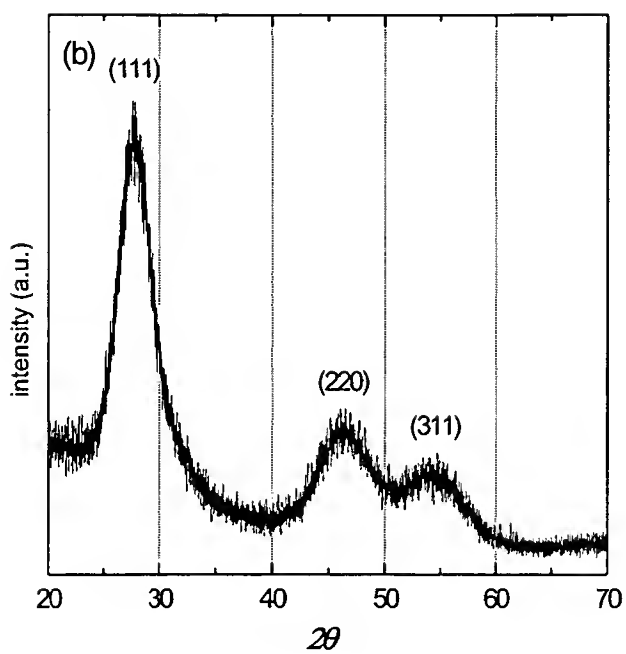
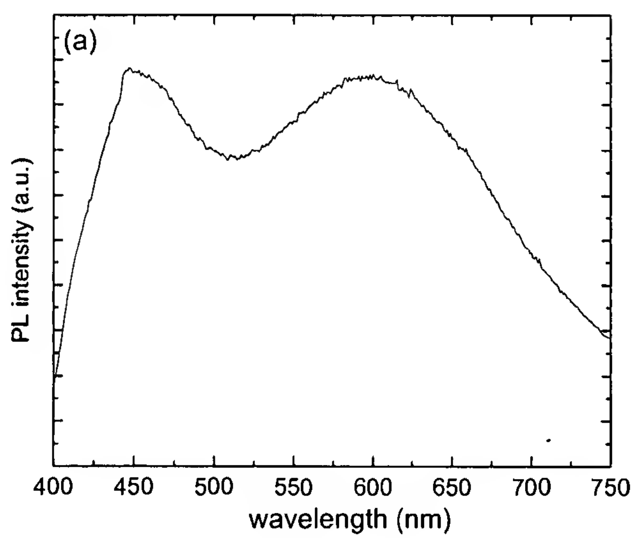
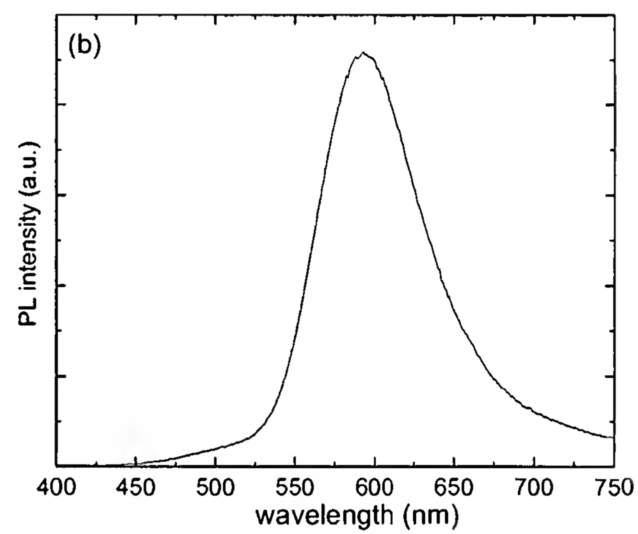


FIG. 2B



(a)
PL intensity (a.u.)
400
450
500
550
600
650
700
750
wavelength (nm)

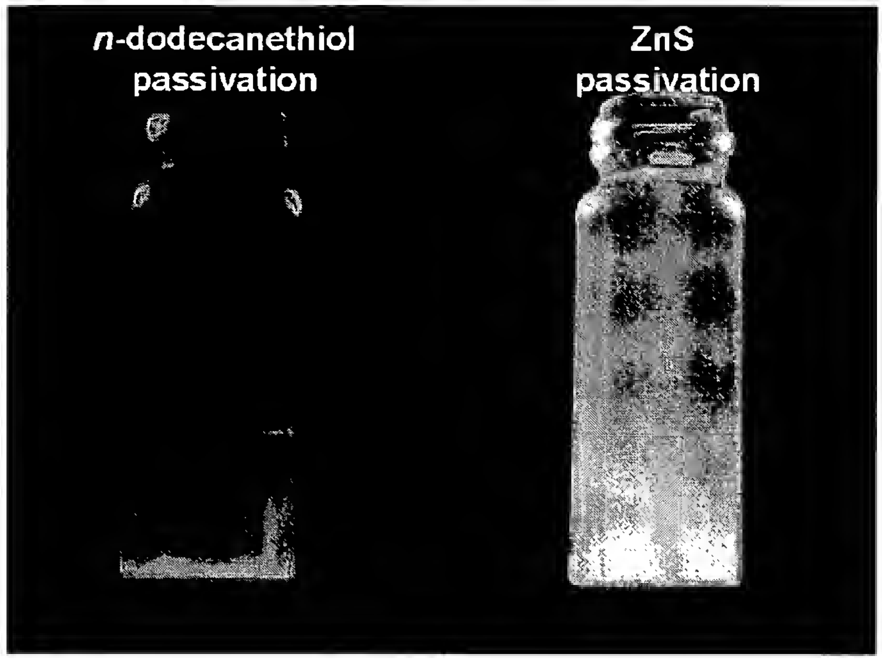
FIG. 3A



(b)
PL intensity (a.u.)
400
450
500
550
600
650
700
750
wavelength (nm)

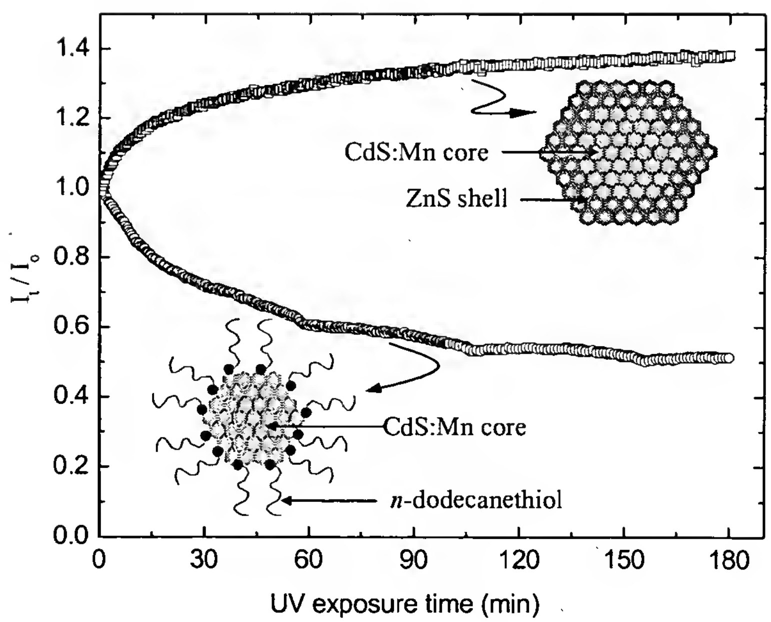
FIG. 3B

FIG. 4



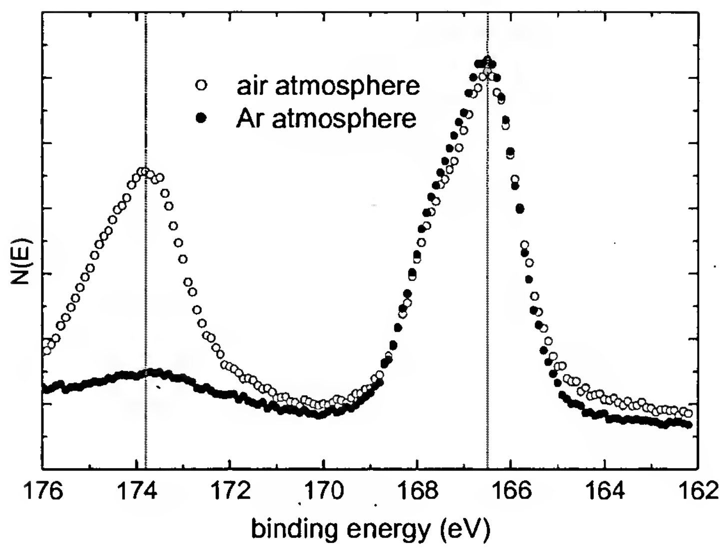
n-dodecanethiol
passivation
ZnS
passivation

FIG. 5



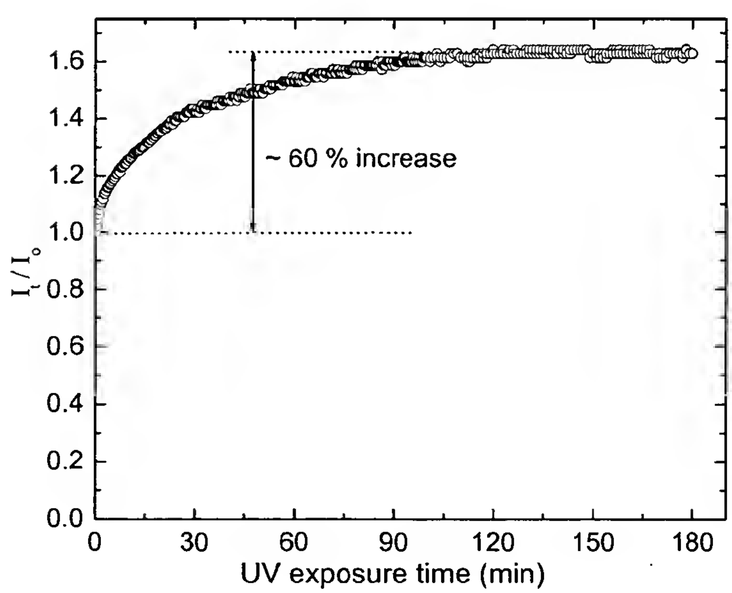
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
I_t / I_o
CdS:Mn core
ZnS shell
CdS:Mn core
n-dodecanethiol
0
30
60
90
120
150
180
UV exposure time (min)

FIG. 6



air atmosphere
Ar atmosphere
N(E)
176
174
172
170
168
166
164
162
binding energy (eV)

FIG. 7



~ 60 % increase
I_t / I_o
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
30
60
90
120
150
180
UV exposure time (min)

FIG. 8A

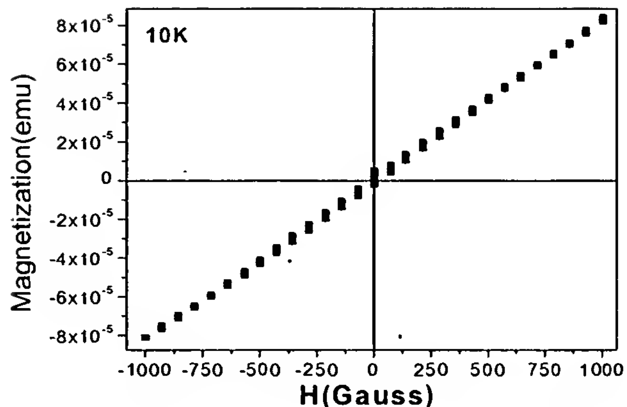


FIG. 8B

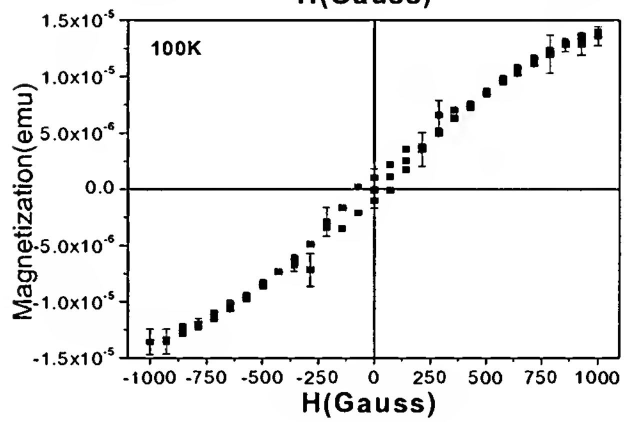


FIG. 8C

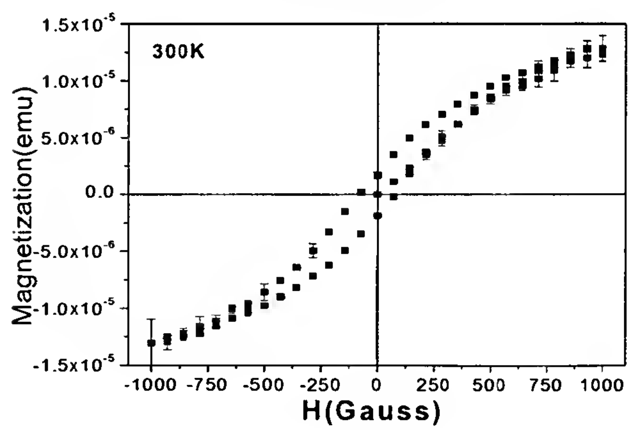


FIG. 9A

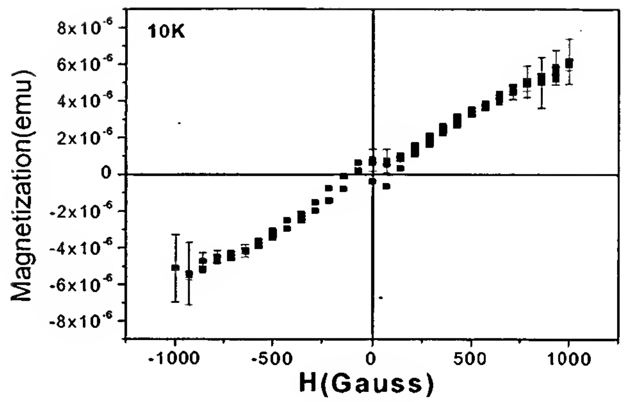

10K
Magnetization(emu)
8x10⁻⁶
6x10⁻⁶
4x10⁻⁶
2x10⁻⁶
0
-2x10⁻⁶
-4x10⁻⁶
-6x10⁻⁶
-8x10⁻⁶
-1000
-500
0
500
1000
H(Gauss)

FIG. 9B

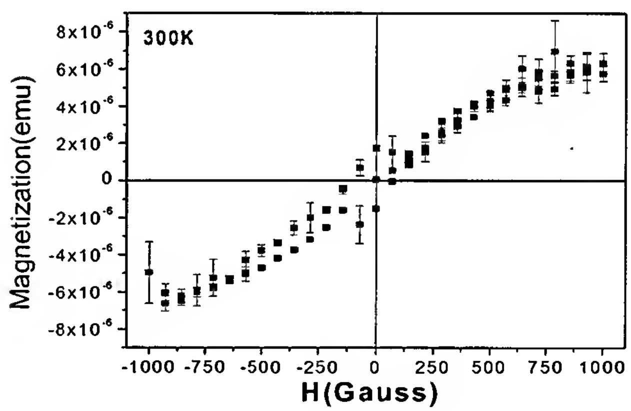

300K
Magnetization(emu)
8x10⁻⁶
6x10⁻⁶
4x10⁻⁶
2x10⁻⁶
0
-2x10⁻⁶
-4x10⁻⁶
-6x10⁻⁶
-8x10⁻⁶
-1000
-750
-500
-250
0
250
500
750
1000
H(Gauss)

FIG. 10A

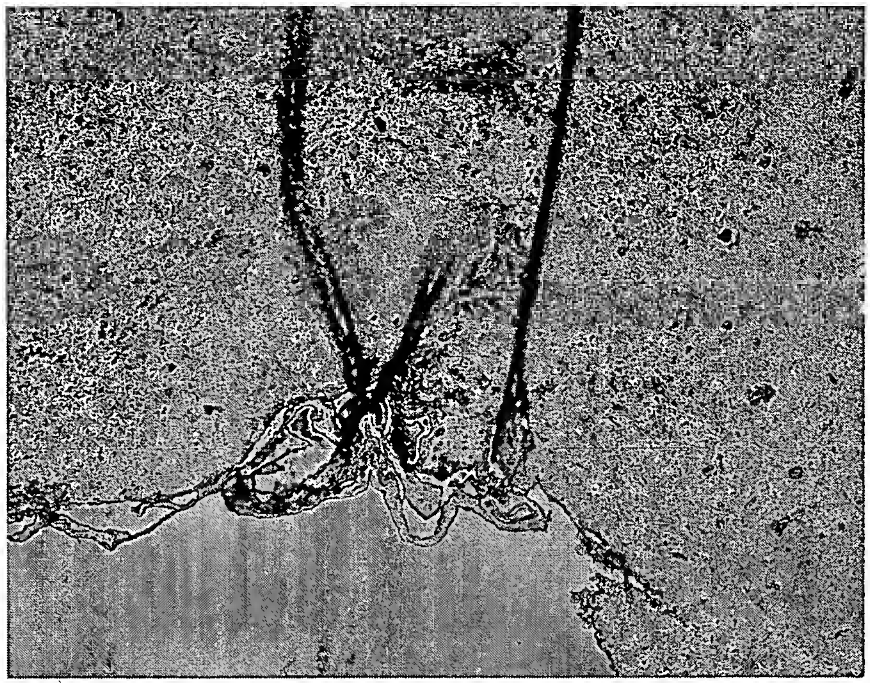


FIG. 10B

FIG. 11

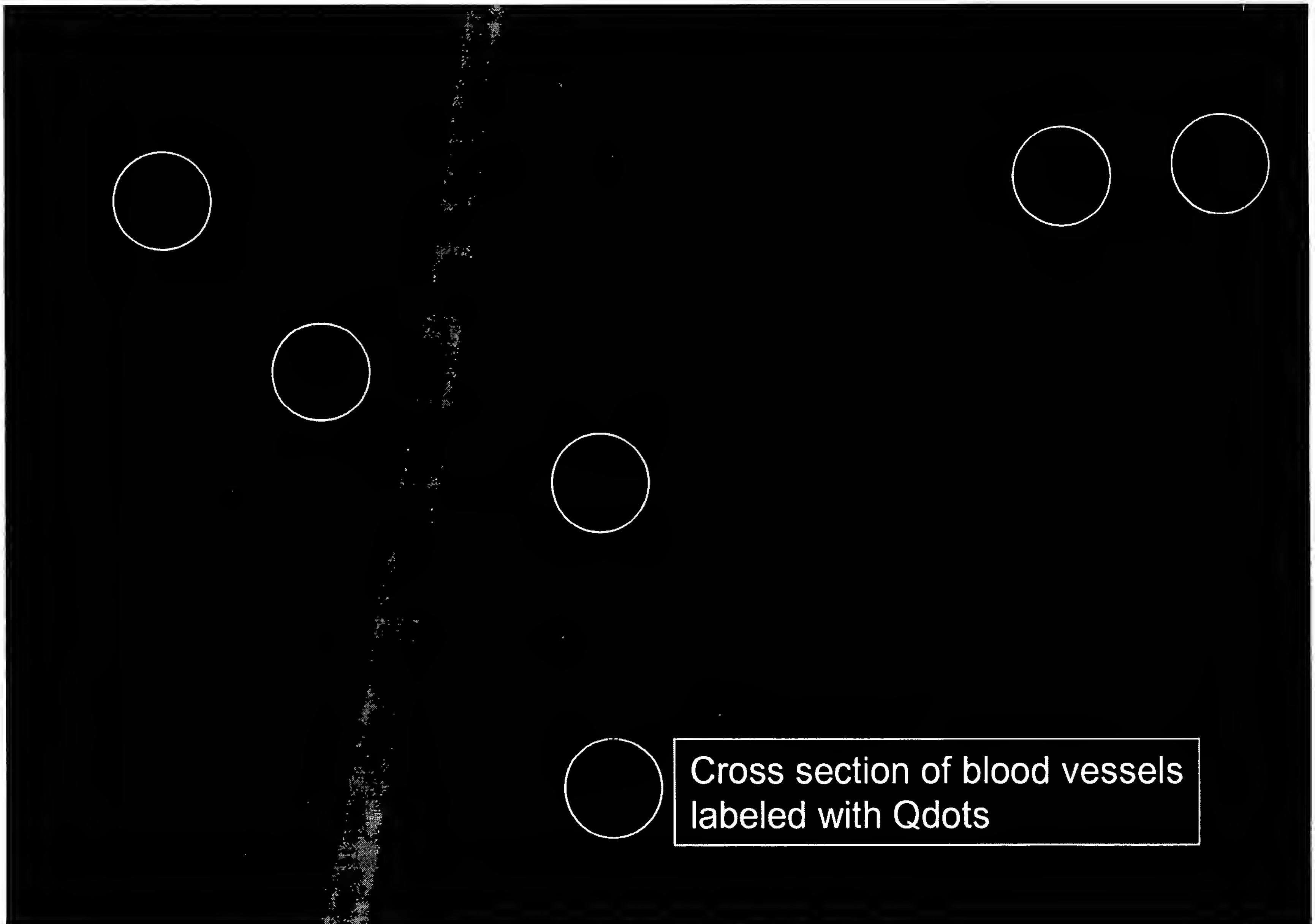


Figure 12